BLOCKING AND REDISTRIBUTION ("CAPPING") OF ANTIGEN RECEPTORS ON T AND B LYMPHOCYTES BY ANTI-IMMUNOGLOBULIN ANTIBODY

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(Received for publication 29 December 1972)

Two types of functionally different lymphocytes may be distinguished in mice by virtue of their surface antigenic markers (reviewed in reference 1). Thymus-dependent (T)[1] cells do not show immunoglobulin (Ig) detectable by immunofluorescence but bear the θ-isoantigen marker. Thymus-independent (B) cells have easily demonstrable immunoglobulin and lack the θ-marker. The mechanism of interaction between lymphocytes and antigens is not clearly understood. It is generally accepted that B cells interact with antigen through specific immunoglobulin surface receptors. In experiments where mouse lymphocyte suspensions were exposed in vitro to [^{125}I]-antigen of high specific activity, it was found that both T and B cells could be specifically killed by the corresponding antigen, pointing toward specific receptors on T cells as well (2–4). This T cell "suicide" could be prevented by pretreatment of the cells with anti-κ chain serum (2), but the nature of T cell receptors is, however, still highly controversial (reviewed in references 5–7).

[^{125}I]Antigen binding to cells can also be visualized by autoradiography (reviewed in references 7 and 8), and it was found that part of the binding cells could be suppressed by treatment with anti-θ serum and complement and represented thus presumably T cells (9). The same observation was made using immunocytoadherance to detect binding cells (10–12).

It appeared to us that the combination of autoradiographic examination of [^{125}I]antigen-binding cells with the detection, on the same cell, of Ig or θ-markers by immunofluorescence would provide a powerful tool to distinguish and study in a direct way specific T and B cells. The nature of the receptor on both types of cells was approached in two ways: (*a*) by using various anti-Ig antibodies to inhibit antigen binding; this approach has been used by others but without the simultaneous direct detection of T or B markers on the binding cell (reviewed

[1] *Abbreviations used in this paper:* ALS, antimouse lymphocyte serum; anti-L, rabbit antimouse L chain; anti-μ, rabbit antimouse μ-chain; antirabbit Ig, sheep antirabbit immunoglobulin; B cells, thymus-independent lymphocytes; Fab-poly-anti-Ig, monovalent Fab fragments of rabbit polyvalent antimouse immunoglobulin; FCS, fetal calf serum; Ig, immunoglobulin; MSH, *Maia squinado* hemocyanin; NRS, normal rabbit serum; poly-anti-Ig, rabbit polyvalent antimouse immunoglobulin; rhod-anti-θ, rhodamine-conjugated anti-θ immunoglobulin G; T cells, thymus-dependent lymphocytes; TIGAL, heavily iodinated (Tyr, Glu)-Ala--Lys; TRITC, tetramethylrhodamine isothiocyanate.

in references 5 and 11); (*b*) by exploiting the recent findings on lymphocyte membrane fluidity (13, 14). Cross-linking of surface immunoglobulins and other membrane-bound antigens induces the formation of microaggregates (spots), which migrate in the plane of the membrane to form caps, followed by endocytosis. Membrane components move independently of each other, e.g., treatment of lymphocytes with anti-Ig sera induces Ig capping but leaves histocompatibility antigens evenly distributed and vice versa. Thus the capacity of various antisera to induce capping of antigen receptors on T cells would be in our opinion a good way to study the nature of these receptors.

Materials and Methods

Antisera—Antitheta C3H serum was obtained in AKR mice (15). Rhodamine-conjugated Ig anti-θ (rhod-anti-θ) was a gift of Dr. F. Loor; the IgG fraction was isolated by agarose block electrophoresis and conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Baltimore Biological Laboratories, Baltimore, Md.) by the dialysis method described by Raff et al. (16). Specificity was ascertained by the absence of staining of AKR mouse thymocytes and nude mouse spleen cells (20×10^6 cells in 0.2 ml plus 0.2 ml of rhod-anti-θ at an Ig concentration of 0.5 mg/ml) as well as by the complete elimination of the capacity to stain C3H lymphocytes by absorption with C3H brain (0.2 ml of rhod-anti-θ at an Ig concentration of 0.5 mg/ml was absorbed with freshly homogenized brain from three mice).

Antimouse lymphocyte serum was prepared by the method of Levey and Medawar (17). It had a 100% cytotoxic activity for thymocytes (5×10^6 cells in a final volume of 0.1 ml) up to a dilution of 1:1,024.

Polyvalent rabbit antimouse immunoglobulin antibody (poly-anti-Ig) and its monovalent Fab fragments (Fab-poly-anti-Ig) were prepared and characterized as described in (14). Poly-anti-Ig was conjugated with TRITC (18). Some rhod-poly-anti-Ig was absorbed twice with 5 mg of unsolubilized mouse IgG myeloma protein per ml serum (RPC-23 IgG1; 5563 IgG2a; MOPC-141 IgG2b) (19). These myeloma proteins were the generous gift of Dr. G. Torrigiani and their preparation and characterization have been described (20). After absorption, this serum no longer stained any spleen cells (20×10^6 cells in 0.2 ml plus 0.2 ml of absorbed serum).

Rabbit antimouse μ-chain antiserum (anti-μ) was a gift from Dr. G. Torrigiani; it was prepared by injection of MOPC-104E mouse IgM myeloma protein isolated from serum (20). Absorption was performed with mouse IgG myeloma proteins (RPC-23 IgG1; 5563 IgG2a; MOPC-141 IgG2b) made insoluble by cross-linking with ethylchloroformate (19). After absorption the undiluted antiserum gave a single line in agarose immunodiffusion when reacted with undiluted normal mouse serum and did not react with mouse myeloma IgG of the different subclasses tested at a protein concentration of 5, 1, and 0.1 mg/ml. In addition anti-μ did not stain surface IgG1 immunoglobulins of P3 myeloma cells[2] (20×10^6 cells in 0.2 ml plus 0.2 ml of anti-μ at a concentration of 0.5 mg Ig/ml revealed with rhodamine-labeled antirabbit Ig), which could be stained by both specific anti-IgG1 and poly-anti-Ig. Anti-μ did not agglutinate sheep red blood cells coated with purified mouse IgG when tested by the method of Gordon et al. (21).

Rabbit antimouse light chain antiserum (anti-L) was a gift from Dr. G. Torrigiani; it was prepared by injection of light chains obtained by reduction and alkylation of purified mouse serum IgG (22). This anti-L, when revealed by rhodamine-labeled sheep antirabbit Ig, reacted with the same number of spleen or lymph node cells as rhod-poly-anti-Ig. It had almost exclusively anti-κ specificity (G. Torrigiani, personal communication).

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[2] Knoff, P. Manuscript submitted to *Eur. J. Immunol.*

Sheep antirabbit immunoglobulin antiserum (antirabbit Ig) was obtained by injection of rabbit Cohn fraction II (Mann Research Labs., Inc., New York) and the Ig fraction was conjugated with TRITC (18). It was used to localize unlabeled anti-μ or anti-L bound on cells by a sandwich technique (14), under which conditions it did not stain mouse spleen cells directly.

Although none of these anti-immunoglobulin reagents stained mouse thymus cells even at an Ig concentration of 2.5 mg/ml, they were nevertheless absorbed with thymocytes (8×10^8 cells/ml for 12 h at 4°C).

Antigens.—Maia squinado hemocyanin (MSH) and heavily iodinated (Tyr, Glu)-Ala--Lys synthetic polypeptide (TIGAL) type 52 (50 atoms of I/molecule) were ^{125}I radioiodinated by the chloramine-T method (3, 9). Specific activity ranged from 150 to 300 μCi/μg. MSH seems to be completely thymus dependent (23) but only the 7S response of TIGAL 52 (J. Humphrey and N. Willcox, personal communication).

Animals.—C3H/He and (A/J $\times$ CBA/J)F_1 mice, 3–6 mo old, were used unprimed or after a single intraperitoneal injection of 100 μg of alum-precipitated antigen (MSH or TIGAL iodinated with nonradioactive iodine) with 10^9 killed *Bordetella pertussis* organisms.

Double Labeling of Cells with [^{125}I]Antigen and Rhodamine Antibody.—Spleen cell suspensions were filtered in ice through cotton gauze to remove clumps, washed twice in Hanks' balanced salt solution, and resuspended at a concentration of 10^8 cells/ml in the appropriate medium (see below).

Conditions for spot and cap formation on lymphocytes, as well as their inhibition, has been described in detail (13, 14). Unless specifically indicated, "noncapping conditions" were used, thus keeping the cells close to 0°C, suspended in 3% bovine serum albumin phosphate-buffered saline, 1.5 mM NaN_3, or, for exposure to [^{125}I]antigen, medium 199 with 10% fetal calf serum (FCS) and 1.5 mM NaN_3.

Exposure of cells to [^{125}I]antigen was as described (9). Briefly, 20×10^6 cells were mixed with 1 μg of [^{125}I]antigen in a total volume of 0.4 ml, maintained on ice for 1 h, spun, resuspended in 0.5 ml, passed through an FCS gradient, and washed once more. The cells resuspended in 0.1 ml were then incubated with 0.2 ml rhod-poly-anti-Ig, anti-μ or anti-L (0.5 mg Ig/ml), or rhod-anti-θ (0.2 mg Ig/ml) for 30 min on ice and washed three times. Rhod-anti-rabbit Ig was used to reveal binding of anti-μ or anti-L.

Inhibition of Antigen Binding by Anti-Immunoglobulin Antibody.—Cells were incubated with normal rabbit serum (NRS), rhod-poly-anti-Ig, anti-μ, anti-L (all at 2.5 mg Ig/ml), or Ig-absorbed rhod-poly-anti-Ig for 30 min in the cold, washed three times, and exposed to the radiolabeled antigen.

Anti-μ and anti-L antibodies fixed on the cell membranes were detected by staining with rhod-anti-rabbit-Ig after exposure to antigen. Inhibition of antigen binding was also tried using antimouse lymphocyte serum (ALS) at a final dilution of 1:100 at which all lymphocytes were very brightly stained with the same ALS conjugated with rhodamine and which did not induce redistribution of surface markers under the usual capping conditions.

Redistribution of Antigen Receptors by Anti-Immunoglobulin Antibody.—To induce capping of immunoglobulin receptors, the cells were incubated with rhod-poly-anti-Ig, anti-μ, and anti-L (all at 0.5 mg Ig/ml) for 30 min in the cold in 199 medium with 10% FCS without NaN_3, washed three times with the same medium, and put at 37°C for 10 min. The cells were then centrifuged, resuspended in NaN_3 containing medium to stop the capping process, and exposed to [^{125}I]antigen under noncapping conditions. The cells treated with unlabeled anti-L were stained with rhod-antirabbit Ig after exposure to antigen, and the cells treated with unlabeled anti-μ were stained with either rhod-antirabbit Ig or rhod-poly-anti-Ig.

Staining with rhod-anti-θ in Inhibition and Receptor Redistribution Experiments.—Preliminary experiments showed that cells that had fixed poly-anti-Ig or anti-L would take up rhod-anti-θ since it is itself mouse Ig. However, since only IgG fractions had been used to make

rhod-anti-θ, they could be used in conjunction with anti-μ antibody in both inhibition and capping experiments. Moreover Dr. F. Loor (personal communication) has shown that monovalent Fab-poly-anti-Ig bound to B cells and cross-linked with a second layer of sheep anti-rabbit Ig did not pick up rhod-anti-θ. Thus rhod-anti-θ was also used after capping induction with Fab-poly-anti-Ig and antirabbit Ig.

Redistribution of Antigen.—The cells were incubated with [^{125}I]MSH for 1 h at 37°C in 199 medium (10% FCS without azide). Incubation with radioactive antigen was also carried out in conditions described to produce a large number of human T rosettes to sheep erythrocytes (24), that is 5 min at 37°C followed by 1 h at 0°C in medium containing 1.2 mM Ca^{++} and 1.0 mM Mg^{++}. As control, cells were preincubated for 5 min at 37°C before addition of antigen. The cells were then studied for Ig or θ under noncapping conditions.

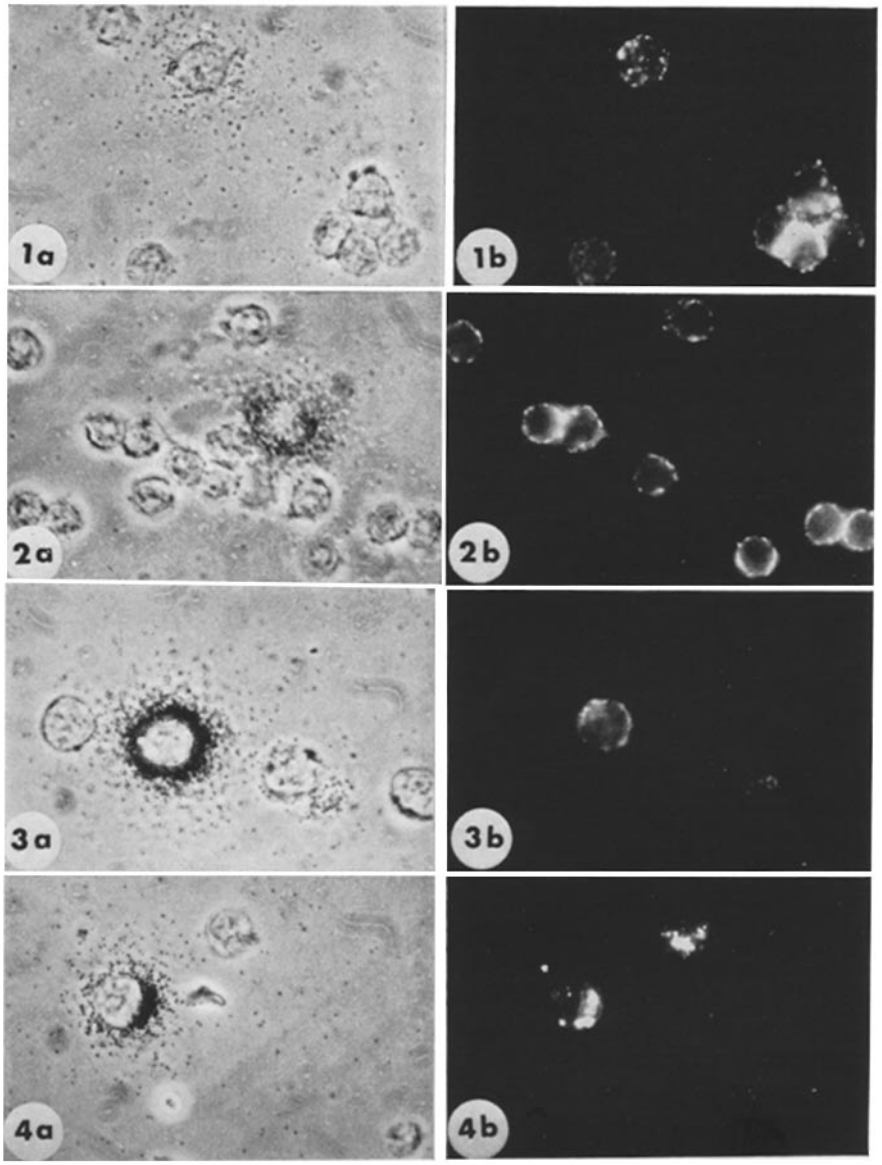
Autoradiography and Microscopy.—Cells were smeared on slides subbed in 0.1% gelatin and 0.01% chrome alum, fixed for 5 min in absolute ethanol at room temperature, processed for autoradiography using Ilford K5 emulsion (Ilford Ltd., Ilford, Essex, England), and developed 2–4 days later (9). The slides were observed under phase contrast at 1,000 magnification with Leitz Orthoplan microscopes equipped with Osram HBO-200 mercury vapor lamps and Opak-Fluor vertical illuminators (E. Leitz, Wetzlar, West Germany) (25). Each antigen-binding cell (20 grains above the background, which in most experiments was negligible) was observed under selective illumination for the visualization of rhodamine (25). To avoid scoring false negatives, the antigen-binding cells apparently negative for surface immunoglobulin were systematically photographed on Kodak TRI-X PAN 23 DIN black-white film (Eastman Kodak Co., Rochester, N. Y.) using 4-min exposure times.

RESULTS

Antigen-Binding B and T Lymphocytes.—Spleen cells of mice primed with TIGAL or MSH were exposed to the appropriate radioactive antigen, washed, and incubated with either rhod-poly-anti-Ig or rhod-anti-θ. All the steps were carried out on ice in the presence of azide, thus under noncapping conditions. Examination of labeled cells showed that there was no significant interference between the silver grains and the fluorescence and that cells could be easily scanned for one and the other (see Figs. 1–4). There was no correlation between the number of grains and the brightness of the fluorescence either by Ig or θ. In all instances antigen grains and Ig or θ-fluorescence were evenly distributed and never in caps.

Since we want to focus on the comparison of B and T binding cells rather than on their precise quantification, and since results obtained with TIGAL and MSH were identical in this respect, they were pooled together in Table I, which represents the mean of 14 concordant experiments.

Primed cells taken 1–2 mo after priming contained more antigen-binding cells (0.38%) than 3.5–4 mo after priming (0.07%). The anti-Ig or anti-θ treatment subsequent to antigen binding did not influence the number of binding cells detected. 1–2-mo primed cells showed a ratio of Ig+ to Ig− and θ− to θ+ antigen-binding cells of about 4:1 that dropped to about 2:1 for 3.5–4-mo primed cells. Although the overall scoring seems to indicate a possible 8% overlap between θ+ and Ig+ antigen-binding cells (22% θ+ cells but only 14% Ig−, 1–2 mo after priming), when the same cell suspension was stained for Ig



Antigen-binding cells. (*a*) Cells unstained in phase contrast. × 1,000. (*b*) Same field in fluorescence selected for rhodamine.

FIG. 1. An Ig+ antigen-binding cell.

FIG. 2. An Ig− antigen-binding cell.

FIG. 3. A θ+ antigen-binding cell.

FIG. 4. A binding cell with coincident polar redistribution of antigen and immunoglobulin.

and θ, the number of Ig+ and $\theta-$ or Ig− and $\theta+$ antigen-binding cells were practically identical. Furthermore, in inhibition and capping experiments presented below, the results were identical whether Ig+ or $\theta-$ cells were examined or Ig− and $\theta+$ cells. Thus, although some overlap cannot be excluded, the bulk of Ig+ and $\theta-$ antigen-binding cells may be considered as B lymphocytes and the bulk of Ig− and $\theta+$ antigen-binding cells as T lymphocytes. Antigen-binding cells of both types can be easily distinguished and studied separately.

Table II shows that within the range we were able to quantitate (60–1,200 molecules per cell), the degree of labeling of B and T cells was identical. This is in contradiction with the conclusions reached from experiments in which indirect evaluation of T antigen-binding cells was done by killing with anti-θ serum and complement (9). Since cells with more than 1,200 molecules started to show confluent grains, it was not possible to determine in a quantitative way if the parallel between T and B cells was also valid for the most heavily labeled cells. All we can say is that very heavily labeled T cells do exist (see Figs. 1–4).

The frequency of antigen-binding cells in unprimed spleens was roughly 0.01%. Heavily antigen-labeled T cells (Ig− or $\theta+$) were seen, but not enough cells were counted to give a valid quantification.

TABLE I

B and T Antigen-Binding Cells in Primed Spleens

	no. of cells counted	Antigen-binding cells*			B cells			T cells	
		no.	% of total		no.	% of total		no.	% of total
1–2 mo after priming	35,471	134	0.38	Ig+	115	86	Ig−	19	14
	44,855	171	0.38	$\theta-$	134	78	$\theta+$	37	22
Total	80,326	305	0.38		249	82		56	18
3.5–4 mo after priming	98,789	75	0.07	Ig+	52	69	Ig−	23	31
	20,075	14	0.07	$\theta-$	11	79	$\theta+$	3	21
Total	118,864	89	0.07		63	71		26	29

* Cells binding more than 60 molecules of antigen.

TABLE II

*Degree of Labeling of T and B Cells Exposed to Radioactive Antigen**

	no. of antigen molecules per cell			
	60–299	300–599	600–1,199	>1,200
	%	%	%	%
Ig+ or $\theta-$	9	22	28	41
Ig− or $\theta+$	5	23	30	42

* Calculated from the number of grains in cells from Table I (9).

Inhibition of Antigen Binding by Anti-Immunoglobulin Antibody.—To try to inhibit antigen binding, the cells were incubated with rhod-poly-anti-Ig, anti-μ, and anti-L at an Ig concentration of 2.5 mg/ml before exposure to [^{125}I]antigen. The specific anti-μ and anti-L sera were prepared by absorption and also absorbed with thymocytes. No unspecific antimembrane activity could be demonstrated (see Materials and Methods). Ig+ cells were detected by the directly conjugated rhod-poly-anti-Ig or by adding, after exposure to [^{125}I]antigen, rhod-antirabbit Ig to cells incubated with anti-L. μ+ cells were also detected by adding, after exposure to [^{125}I]antigen, rhod-antirabbit Ig to cells incubated with anti-μ.

It was found in preliminary experiments that anti-θ antibody, being itself of mouse origin, was picked up by cells having bound poly-anti-Ig or anti-L antibodies. However, since only IgG fractions had been used to make rhod-anti-θ antibody, it could be used to stain the cells where inhibition had been tried with anti-μ.

The inhibition of antigen binding was calculated by comparison with the number of antigen-positive cells found in the same cell suspension by treatment with [^{125}I]antigen followed by rhod-poly-anti-Ig. Normal rabbit serum with an Ig concentration approximately identical with that of the various anti-Ig preparations served as control. Even when not absorbed with cells it did not inhibit antigen binding.

The results are shown in Table III. For antigen-binding B cells, poly-anti-Ig inhibited 78% and anti-L 91%. After treatment with anti-μ the cells were stained with either rhod-poly-anti-Ig detecting all the Ig+ cells or rhod-anti-rabbit Ig detecting only the μ+ cells. Anti-μ inhibited 50% of the Ig+ or θ— antigen-binding cells and 80% of the μ+ antigen-binding cells. One should thus be careful here to distinguish inhibitory efficiency from reactivity. Higher concentrations were not used to see if all the cells could be inhibited. Binding to T cells was practically completely inhibited by poly-anti-Ig, anti-L, and also

TABLE III

Inhibition of Antigen Binding by Anti-Immunoglobulin Antibody

Antibody‡	Inhibition of antigen-binding cells*	
	Ig+ or θ−	Ig− or θ+
	%	%
Poly-anti-Ig§	78 (96–55)	>95
Anti-L	91	>97
Anti-μ‖	50 (66–33)	>95
NRS	No inhibition	No inhibition

* Concordant results were obtained with TIGAL and MSH.

‡ 2.5 mg Ig/ml.

§ Mean of three experiments.

‖ Mean of five experiments.

anti-μ. Preliminary experiments with more diluted antibody also showed 60% inhibition of T antigen-binding cells at concentrations giving only at most 10% inhibition of B antigen-binding cells. Thus it appears that binding of antigen to T cells is more readily inhibited by anti-immunoglobulin antibody than to B cells. The T antigen-binding cells, unlike B cells, were completely inhibited by anti-μ as well as anti-L, suggesting that T receptors for antigen are IgM. In contrast to anti-Ig, ALS did not inhibit antigen binding by B or T cells (Table IV).

In some experiments, cells were exposed to anti-θ before or together with [^{125}I]antigen. No inhibition of binding was observed.

Redistribution of Antigen Receptors by Anti-Immunoglobulin Antibody.—Capping of the surface Ig of B lymphocytes can be induced by anti-Ig antibody and followed by fluorescence (13, 14). It was argued that (*a*) if these Ig's were the

TABLE IV

*Inhibition of Antigen Binding by Antilymphocyte Serum**

	Control	ALS
No. of cells counted	23,918	22,008
No. of antigen-binding cells	95	87
Total	0.40%	0.39%
Ig+	80%	78%
Ig−	20%	22%

* Cells were incubated with ALS or NRS, washed, and treated with [^{125}I]TIGAL followed by rhod-poly-anti-Ig.

receptors for [^{125}I]antigen, the silver grains should be seen in caps superimposed on the Ig fluorescent caps; and (*b*) if the receptors on T cells were indeed immunoglobulins, as suggested by the inhibition experiments, they would also be induced to cap by anti-Ig antibody. Consequently, on T cells as on the B cells, the [^{125}I]antigen grains would appear in caps.

Capping was induced by poly-anti-Ig, anti-μ, anti-L, or Fab-poly-anti-Ig combined with antirabbit Ig at an Ig concentration of 0.5 mg/ml that produced no discernible inhibition of antigen binding. The cells were then washed, brought to noncapping conditions, exposed to [^{125}I]antigen, and stained for the Ig or θ-markers (see Materials and Methods).

Since cells do not cap synchronously after treatment with anti-Ig, some cells will already start endocytosis while others do not yet show an apparent redistribution. In order not to score false Ig or antigen-negative cells, capping was stopped after 10 min, at which time no significant endocytosis had yet occurred. Thus all stages of Ig or antigen redistribution could be seen from complete concentration on one pole of the cell to still apparently even distribution over the whole surface of the cell. Ig's were scored as "capped" when present over less

than half of the cell, and antigen when there was a markedly asymmetrical concentration of the grains. Due to scattering, a complete absence of grains on half of the cells was not always obtained (see Figs. 1–4).

The results were similar with the various combinations of reagents used and are presented together in Table V. 83% of the Ig+ cells had coincident antigen grains and Ig fluorescence, 57% had superimposed caps; 26% were still evenly distributed; 15% had the Ig fluorescence in cap but not the antigen, probably indicating still incomplete capping of the Ig receptors on those cells. Four cells were found with the antigen in cap but not the Ig. Of crucial significance, antigen was detected in caps on 54% of the T cells (Ig− or θ+), a proportion very close to that found for B cells.

TABLE V

*Anti-Immunoglobulin-Induced Redistribution of Antigen Receptors on B and T Cells**

	Antigen	Immunoglobulin	Antigen-binding cells		no. of cells exam ned
			no.	% of total	
Ig+ cells	Noncapping	Noncapping	89	26	
	Capping‡	Capping‡	195	57	
	Noncapping	Capping	52	15	
	Capping	Noncapping	4	1	307,000
Ig− or θ+ cells	Noncapping		50	46	
	Capping		59	54	345,000

* Compiled from nine experiments using poly-anti-Ig, anti-μ, anti-L, or anti-Fab antibody under capping conditions followed by [^{125}I]antigen and the appropriate rhod-anti-Ig or rhod-anti-θ under noncapping conditions. Concordant results were obtained with TIGAL and MSH.

‡ Cells were scored as capped when they had Ig or antigen concentrated in less than half of the cell surface.

The antigen-capped θ+ cells still had the θ-fluorescence diffuse as usual, showing again the specificity of capping induction. Significant capping was not induced in B or T cells by treatment with NRS or Ig-absorbed rhod-poly-anti-Ig (Table VI).

Since capping of antigen receptors on T cells was induced by four well-characterized preparations as different as poly-anti-Ig, anti-μ, anti-L, and Fab-poly-anti-Ig that had all been extensively absorbed with thymocytes, and since significant capping was not induced by NRS or Ig-absorbed poly-anti-Ig, it is extremely improbable that all the antisera would contain antibodies against a T receptor that would not be immunoglobulin but be absorbable by Ig preparations. Since in addition the rate of capping was identical in T and B cells, one is forced to conclude that by far the most likely explanation is that the T receptors for TIGAL and MSH are immunoglobulin.

Redistribution of Antigen.—We wanted to investigate whether cross-linking

TABLE VI

*Specificity of Anti-Immunoglobulin-Induced Redistribution of Antigen Receptors on B and T Cells**

	Antigen	Immunoglobulin	Antigen-binding cells‡					
			rhod-poly-anti-Ig		Normal rabbit serum		Ig-absorbed rhod-poly-anti-Ig	
			no.	% of total	no.	% of total	no.	% of total
Ig+ cells	Noncapping	Noncapping	20	22	97	99	73	88
	Capping§	Capping§	64	71	0	0	3	4
	Noncapping	Capping	6	7	0	0	1	1
	Capping	Noncapping	0	0	1	1	6‖	7
Ig− cells	Noncapping		10	31	17	94	22	96
	Capping		22	69	1	6	1	4

* Rhod-poly-anti-Ig, NRS, or Ig-absorbed rhod-poly-anti-Ig used under capping conditions followed by [^{125}I]TIGAL and rhod-poly-anti-Ig (for the last two groups) under noncapping conditions.

‡ 60,000–70,000 cells were counted per group.

§ Cells were scored as capped when they had Ig or antigen concentrated in less than half of the cell surface.

‖ Those six cells were all very low antigen binders (60–130 molecules).

TABLE VII

*Redistribution of MSH on B and T Cells**

	Antigen	no. of antigen-binding cells	Decrease in no. of antigen-binding cells vs. controls
Ig+ or θ−	Noncapping	43	None
	Capping‡	44	
Ig− or θ+	Noncapping	3	69%
	Capping‡	2	

* [^{125}I]MSH under capping conditions followed by rhod-poly-anti-Ig or rhod-anti-θ under noncapping conditions.

‡ Cells were scored as capped when they had antigen concentrated in less than half of the cell surface.

of receptors by antigen itself would cause capping on B and T cells as well. Cells were exposed to [^{125}I]MSH and kept for 1 h in capping conditions, washed, and incubated with either rhod-poly-anti-Ig or rhod-anti-θ under noncapping conditions. The results are presented in Table VII. About 50% of the B cells had the antigen in caps, but all still had Ig distributed over the whole surface, indicating that the concentration of antigen used (2.5 μg/ml) was not saturating the receptors. There was no decrease in the number of B antigen-binding cells compared

with controls. On the contrary, only 31% of the expected T antigen-binding cells were found.

Conditions described by Jondal et al. (24) to produce a large number of human T rosette-forming cells for sheep erythrocytes were also used to see if the number of T or B binding cells or the capping pattern with antigen would be modified. Cells were incubated with [^{125}I]MSH for 5 min at 37°C in 199 medium (1.2 mM Ca^{++}, 1.0 mM Mg^{++}, 10% FCS), cooled, and incubated further in ice for 1 h. Cells were also first incubated 5 min at 37°C by themselves and the antigen added when they were brought to 0°C. They were then stained with rhod-anti-θ or rhod-poly-anti-Ig. These treatments had no influence on the number of binding cells nor did they induce capping on either T or B cells. The kinetics of capping of antigen on T and B cells is being further investigated.

DISCUSSION

The main scope of our work was to establish the existence of antigen-binding T lymphocytes and to collect information on the nature of antigen-binding receptors on these cells. Our approach was based on the identification of each cell that had bound radioactive antigen as B or T on the basis of its reactivity with fluorescent anti-θ or fluorescent anti-Ig. Our assumption was that a T cell would be identified by immunofluorescence as θ positive or as immunoglobulin negative, whereas the reverse would happen in the case of a B cell. This assumption is grounded on several previous observations (reviewed in reference 1) and is valid provided that some potential errors or limitations are duly taken into account, both with regard to the identification of T on the basis of θ positivity and of B on the basis of reactivity with anti-Ig serum.

For the first kind of identification the main problem is the specificity of the anti-θ antiserum employed. When prepared in other than a congenic strain of mice, antibodies of unwanted specificity may be present, especially antiallotype antibodies (26), autoantibodies (27), and others (28).

After conjugation with rhodamine, the isolated IgG fraction of the anti-θ serum did not stain AKR mouse thymocytes nor nude mouse spleen cells. Moreover, its capacity to stain C3H thymocytes or spleen cells was completely removed by absorption with C3H brain (see Materials and Methods). Thus it appeared that, although not prepared in a congenic strain of mice, the rhod-anti-θ stained only T cells.

On the other hand, the identification as B cells of cells positive in immunofluorescence with anti-immunoglobulin antisera is not only subject to the specificity of the antisera employed, but is also in apparent contradiction with the statements of several workers (11, 29–33) and even with the conclusion of our own work reported here, namely that there are immunoglobulins on the surface of the T lymphocytes. What one can say in this respect is that these molecules are not easily demonstrable by immunofluorescence, and in fact, were not seen under the conditions employed in the present work.

The combined percentages of $\theta+$ and Ig+ cells in a given sample were always very close to 100. In addition, throughout this work very similar results were obtained irrespective of whether T cells were scored as $\theta+$ or Ig− and B cells as $\theta-$ or Ig+. Therefore we are confident that we have indeed been studying the antigen-binding properties of two separate populations of mouse spleen lymphocytes corresponding to the thymus-dependent (T) and to the thymus-independent (B) cells. They will therefore be discussed separately.

Antigen-Binding B Lymphocytes.—With the two antigens used, the B antigen-binding cells were always more numerous than the T antigen-binding cells, about 80% 1–2 mo after priming and about 70% 3–4 mo after priming; nevertheless, in none of our experiments were the B cells the only antigen-binding cells; therefore, the conclusion of Warner (34) and Lamelin et al. (35) that demonstrable [^{125}I]antigen binding is an exclusive property of B cells does not seem to be generally valid.

Under the conditions used, our attempt to quantitate the number of antigen molecules bound by individual B lymphocytes could only resolve the low end of the spectrum, since beyond the level of approximately 1,200 molecules per cell, the grains became confluent and could not be counted (Table II). Interestingly there was no correlation between the number of antigen molecules bound by a given B cell and the intensity of the anti-immunoglobulin staining; thus it is possible that the affinity and not the number of the receptors could be the main factor in determining the variability in the number of antigen molecules bound by different B cells.

When capping of B cell immunoglobulins was induced by anti-immunoglobulin antibodies used at a concentration insufficient to inhibit antigen binding (0.5 mg Ig/ml), as a rule the antigen bound was detected at a site corresponding to the Ig (Table V). However, in a small but significant proportion of B cells (15%) this did not happen, and the result was that although immunoglobulins localized by immunofluorescence appeared to be in cap, the antigen molecules demonstrated by autoradiography were still apparently uniformly distributed around the cell. The reason for this unexpected finding is still not elucidated, but it might be that those were B cells with high affinity receptors (they were indeed all in the high grain count range) and that in those cells incomplete capping of the surface immunoglobulins left behind a number of receptors insufficient to be detected by immunofluorescence but sufficient to bind antigen molecules, whereas in the immunoglobulin cap the packing of the immunoglobulin–anti-immunoglobulin complex did not allow an appreciably higher density of bound antigen molecules; thus the fluorescent Ig would appear as a cap while the antigen grains would not.

It would not be surprising if for cells with high affinity receptors and for antigens possessing many identical determinants, like those used, the antigen/antibody ratio was different in a cap as compared with areas of the cell where receptors are more sparse. The demonstration by autoradiography of antigen

bound in areas of B cells where immunoglobulins are not demonstrable by immunofluorescence underscores the difference in sensitivity of the two techniques and is an element to be remembered when discussing the nature of the antigen-binding receptors of T cells.

Good inhibition of antigen binding to B cells was obtained by pretreating the cells with a polyvalent antimouse Ig or an antimouse L chain antiserum used at a concentration of 2.5 mg Ig/ml. On the other hand, only about one half of the antigen-binding B cells were inhibited by a comparable concentration of an antimouse μ-antiserum. This discrepancy was presumably due to the presence in primed mice of an appreciable proportion of antigen-specific B lymphocytes with receptors of other immunoglobulin classes.

Experiments in which MSH was allowed to redistribute in polar caps (Table VII) demonstrated that, at the concentration of antigen used, the B binding sites were not saturated, as evidence by the persistence of Ig fluorescence all around the cells. The same result was also obtained using polymerized flagellin by M. Raff (personal communication). However, he was also able to demonstrate that higher doses of the same antigen could completely carry along all the Ig in the antigen cap.

Antigen-Binding T Lymphocytes.—Indirect techniques using radioactive antigen have provided negative (34, 35) and positive (9, 36) evidence for T antigen-specific binding cells. By the simultaneous double-labeling technique used here the existence of these cells was directly demonstrated confirming the results of T cell suicide experiments (2–4).

Lamelin et al. (35) also used labeling with [^{125}I]antigen and fluorescent antisera but did not detect antigen-binding T cells. This may be due to differences in techniques, mainly the use of antimouse thymocyte serum to identify T cells and of sandwich fluorescent techniques exclusively. Antimouse thymocyte serum is notoriously difficult to characterize, and its ability to detect all and only T cells very controversial. Sandwich procedures with some antisera, including the poly-anti-Ig used in the present study, have in our hands been found to displace most of the bound antigen from the surface of the cell. We are studying further if this displacement is easier for T cells.

The absolute numbers of T antigen-binding cells, as well as their ratio to the number of B antigen-binding cells, were very similar for both antigens used, but this should not be generalized to all antigens and consequently one should not conclude that antigen-binding T cells, as a group, are inferior in number to the antigen-binding B lymphocytes; in fact, ratios of T and B sheep red cell-binding lymphocytes have been reported to fluctuate considerably during the course of immunization (12).

The number of antigen molecules bound by each T lymphocyte appears to vary within a wide range with a distribution very similar to that of B cells (Table II); by analogy with B lymphocytes it seems probable that affinity rather than the number of receptors is the major factor in determining the

number of antigen molecules bound per T cell as well. There is a considerable number of T cells that bind more than 1,200 molecules (Table II); therefore, in considering that both antigens employed had multiple identical antigenic determinants, one may conclude that there are T cells that have a number of antigen-binding receptors ranging in the order of thousands. Experiments are in progress to extend the comparative quantitative analysis of antigen bound by T and B lymphocytes to cells that bind more than 1,200 molecules of antigen.

Probably the most interesting part of this work concerns the effect of anti-immunoglobulin antibodies on antigen binding by T cells. There was practically complete inhibition of antigen binding on T cells by pretreatment with anti-mouse immunoglobulin (polyvalent), antimouse L chain, and antimouse μ-chain (all 2.5 mg Ig/ml). Furthermore, when the lymphocytes were pretreated with subinhibiting concentrations of antimouse immunoglobulin antibodies under conditions that induced about 50% of the B cells to display superimposed Ig and antigen caps, a comparable proportion of T cells also bound the antigen as a cap (Table V). The latter effect was not obtained when the antimouse immunoglobulin antiserum was used after absorption with a purified preparation of mouse immunoglobulin different from the preparation that had been employed to raise the antiserum (Table VI). Together with the lack of any significant effect of normal rabbit serum, this supports the conclusion that the capping of receptors for antigen in our T cells was induced through the specific effect of anti-immunoglobulin antibodies.

In spite of the reassuring observation that the effect of anti-immunoglobulin antisera on T cell receptors for antigens was abolished by absorption of the antisera with purified immunoglobulins, the possibility was still considered that this might have been due to some other antibodies present in the antisera directed against nonimmunoglobulin components of the lymphocyte membrane. While this possibility is difficult to rule out completely, it is made unlikely by the fact that an antiserum rich in such antibodies (an antilymphocyte antiserum that reacted strongly in direct immunofluorescence with the membranes of all mouse lymphocytes) did not significantly inhibit the antigen binding by either T or B cells (Table IV).

On the whole, our conclusion is that the antigen-binding moiety on the surface of a T cell is immunoglobulin or a molecule closely associated in the membrane with immunoglobulin. It is clear that the former possibility, as in the case of B cells, is by far the simplest and therefore the most likely one. In addition, the almost complete inhibition of antigen binding to T cells by antiserum specific for mouse μ-chains indicates that antigen-binding immunoglobulin molecules of T lymphocytes are probably IgM. Interestingly enough, no difference was found in antigen binding by T (or B) cells when the cells were exposed to the antigen for 5 min at 37°C and then in the cold, instead of in the cold only. This observation, which is not unexpected if the antigen-binding molecule is an immunoglobulin for both B and T cells, clearly separates the antigen binding by T cells

from events that require cell membrane mobility like those involved in the non-immune adhesion of sheep erythrocytes to human T lymphocytes (24). On the other hand, when lymphocytes were exposed to MSH for 1 h at 37°C in absence of NaN_3, T and B cells behaved in a different way; the T cells tended to lose the antigen much more quickly either by shedding of the receptor or by endocytosis followed by rapid digestion of antigen and elimination of the radioactive marker. We are now studying these events by observing the cells at intervals shorter than 1 h.

Having reached the conclusion that antigen-binding molecules of T lymphocytes are immunoglobulins, it remains to be proved that they are the active products of the cells that carry them, and are not passively acquired, for instance as cytophilic antibodies. The latter possibility is practically excluded by the very fact that antigen-binding cells were always a small minority of the total T cell population, but one might consider the possibility that antigen molecules bound in vivo or in vitro by nonimmunoglobulin T receptors might be binding soluble antibodies and that these, in turn, might bind more radioactive antigen. This possibility is extremely unlikely if one considers the procedures employed in the binding and in the inhibition experiments, as well as the long period of time between the injection in the animals of antigens that are known to be rapidly cleared in vivo and the collection of the cells. Therefore, through the functional tests used in this series of experiments, it can be concluded that the antigen-binding receptors of T lymphocytes are immunoglobulins.

This conclusion is in agreement with the findings of others using immunocytoadherence (11), electron microscopy (29), cytotoxicity (30), or chemical approaches (31–33) to identify T cell receptors. There are, however, important differences between the membrane-bound immunoglobulin receptors of T and B cells in that the former bind much less anti-immunoglobulin antibodies than the latter.

The fact that Ig was not detected by fluorescence even on heavily labeled antigen-binding cells or when the antigen was seen in cap remains largely unexplained. It is well known that T cells bind much less anti-immunoglobulin than B cells; thus it is not usually possible to visualize T cell membrane immunofluorescence by staining with fluorochrome-conjugated anti-immunoglobulin antisera as used in our experiments (reviewed in reference 1). One factor may be that T cells have fewer Ig receptors than B cells, and that we were only detecting those of higher affinity; thus they would be closer to saturation and give detectable antigen binding by autoradiography but no detection by fluorescent anti-Ig. On the other hand, T cell Ig receptors may have a different kind of association with the cell membrane rendering them less accessible to anti-Ig sera (11, 29).

Finally, we wish to point out that these experiments are concerned exclusively with the binding by T and B cells of soluble antigen molecules, but that cell triggering involves further steps yet unclear that probably involve in the case

of T cells the product(s) of Ir genes (37). Moreover, they do not exclude that for interaction with antigen on surfaces (such as membranes of other cells) the T lymphocyte may have ways and means of specific recognition that are different from, or possibly additional to, the immunoglobulin molecules.

SUMMARY

Antigen-binding T and B lymphocytes were studied by combined autoradiography and immunofluorescence; mouse spleen lymphocytes binding the antigens, [^{125}I]MSH or [^{125}I]TIGAL, were incubated with rhodamine-labeled anti-Ig reagents or with a rhodamine-labeled IgG fraction of anti-θ serum. B cells were identified as Ig+ or $\theta-$, T cells as Ig− or $\theta+$.

It was found that: (*a*) 20% (1–2 mo after priming) to 30% (3.5–4 mo after priming) of the antigen-binding cells were T cells. (*b*) The range of antigen molecules bound by B and T cells was similar. (*c*) Binding of antigen to B and T cells was inhibited by polyvalent anti-Ig, anti-μ, or anti-L reagents. Binding to T cells was more readily inhibited than to B cells. Normal rabbit serum, antimouse lymphocyte serum, or anti-θ did not inhibit antigen binding. (*d*) When Ig at the surface of B cells was induced, by noninhibiting concentrations of anti-Ig reagents, to redistribute into polar caps and the cells subsequently exposed to [^{125}I]antigen under noncapping conditions, the [^{125}I]antigen silver grains were distributed in caps superimposed on the Ig fluorescent cap. Of crucial importance, antigen was found in cap in the same proportion of T cells as B cells. Significant capping of antigen receptors was not induced in B or T cells with normal rabbit serum or by anti-Ig reagents absorbed with mouse Ig.

The main conclusions of this series of experiments using direct visualization of antigen-binding B and T lymphocytes is that T cells have antigen-specific receptors, probably of IgM nature, and that the number of these receptors appears to range in the order of thousands.

We are greatly indebted to Dr. F. Loor for the conjugation of rhodamine-labeled anti-θ immunoglobulins and to Dr. G. Torrigiani for the gift of various antimouse immunoglobulin preparations. We thank Dr. J. W. Goodman and Dr. J. R. Kettman for reviewing the manuscript and Miss A. Ryden for outstanding technical assistance.

Noted Added in Proof.—Since this work was accepted for publication, we have studied the effect of decreasing the concentration of [^{125}I]antigen on the labeling of B and T lymphocytes. At an antigen concentration similar to that used by Lamelin et al. (35), that is 100 times lower than the one used in this paper, the number of antigen-binding cells detected was about 10 times lower and the degree of labeling weak (below 50 grains). Moreover only very rare T binding cells were seen. Thus, there is no contradiction between the results reported here and those of Lamelin et al. (35).

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